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Mestrado em Ecologia Marinha

Sofia dos Reis Alves e Dias Francisco

Dissertação orientada por:

Professor Doutor Rui Rosa
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Doutor António Marques
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Resumo

Na época pré-industrial as concentrações de dióxido de carbono (CO_2) atmosférico eram aproximadamente 280 ppm e atualmente excedem os 390 ppm. É estimado que no ano 2100 as concentrações de CO_2 atmosférico cheguem aos 420 a 940 ppm. Este aumento tem provocado um aumento global da temperatura dos oceanos, que até 2100 é esperado aumentar entre 0,3 °C e 4,8 °C, especialmente nas zonas costeiras. Este aquecimento afetará a performance dos animais marinhos em diferentes estados do ciclo de vida através de mudanças na sua fisiologia.

Simultaneamente, os oceanos estão a absorver CO_2, alterando o seu balanço químico, causando uma diminuição no pH dos oceanos e provocando assim, a acidificação dos oceanos. Desde a época pré-industrial, o pH da superfície do oceano diminuiu cerca de 0,1 unidades e é esperado que diminua entre 0,13 a 0,42 unidades até 2100. No entanto, estes valores poderão ser mais elevados em zonas costeiras. Esta acidificação irá afetar diversos organismos como bivalves e peixes. Uma das alterações que este aumento de CO_2 poderá envolver nos peixes é um dispêndio energético acrescido para acomodar as necessárias adaptações fisiológicas.

Além do aquecimento e acidificação dos oceanos, os organismos marinhos têm ainda que lidar com outro fator de *stress* existente: a contaminação. Os ecossistemas marinhos estão a ser contaminados com diversos compostos tóxicos, como os metais. Um destes metais é o mercúrio que é libertado para o ambiente através de fontes naturais ou antropogénicas. Depois da revolução industrial, as emissões de mercúrio antropogénico aumentaram drasticamente e as previsões apontam para a continuação deste aumento. Atualmente as emissões de mercúrio são maioritariamente de origem antropogénica e parte das mesmas são depositadas no oceano. A transformação de mercúrio em metilmercúrio ocorre através de bactérias existentes nos sedimentos. O metilmercúrio bioacumula em organismos aquáticos e biomagnifica ao atravessar os diferentes níveis tróficos, aumentando assim a sua toxicidade e chegando a níveis muito elevados nos organismos que ocupam o topo da teia trófica. Para além disso, pode causar efeitos adversos nos peixes, como por exemplo, alterar a performance reprodutiva, aumentar o *stress* oxidativo e alterar a atividade enzimática do sistema nervoso central.

Os organismos marinhos terão de lidar com os efeitos do aquecimento, acidificação e contaminação dos oceanos simultaneamente. No entanto, pouco se sabe sobre as interações entre estes fatores nos seres vivos. Alguns estudos mostram que os impactos das alterações climáticas nas espécies marinhas podem ser agravados pela

poluição e que as interações entre a temperatura e a poluição por metais influencia a tolerância fisiológica de animais aquáticos ectotérmicos. Além disso, a acidificação do oceano também pode intensificar a toxicidade dos metais.

Com o objetivo de avaliar as respostas biológicas de corvinas juvenis (*Argyrosomus regius*), em relação à exposição ao aquecimento dos oceanos (+ 4 ºC) e acidificação (ΔpH = 0,5 unidades) e ao MeHg (8.02 mg kg^{-1} dw), foi determinado o impacto combinado destes fatores sobre o índice de condição de Fulton, grau de encefalização, níveis de acetilcolinesterase, resposta ao choque térmico, peroxidação lipídica e atividade das enzimas antioxidantes. É importante referir que *A. regius* é um peixe resistente e pode facilmente adaptar-se às alterações ambientais, no entanto, esta espécie é encontrada em zonas costeiras e estuários, especialmente na fase juvenil, onde é expectável que as alterações climáticas irão ter maior impacto no futuro. Sendo a corvina um peixe de elevado valor comercial, é importante avaliar os efeitos futuros que estes fatores de *stress* terão sobre esta espécie.

Os resultados indicam que o aquecimento, a acidificação e a contaminação de mercúrio promovem diversas alterações fisiológicas. A bioacumulação de mercúrio foi afectada principalmente pelo aumento da temperatura, sendo o fígado o tecido que acumulou mais mercúrio. No entanto, não ocorreram diferenças na taxa de sobrevivência e na condição de Fulton. Relativamente à taxa de encefalização, apenas foram observadas alterações nos organismos sujeitos a aquecimento e hipercapnia, onde o tronco cerebral aumentou sob a condição de aquecimento e pH atual.

Foi observado um aumento em ambas as condições controlo de temperatura (18ºC) e pH (8.0) na resposta das proteínas de choque térmico. No entanto, ocorreu uma diminuição significativa destas proteínas, sob condições de aquecimento e hipercapnia. Em oposição, os níveis de malondialdeído observados não mostraram diferenças significativas entre os tratamentos. Relativamente às enzimas de *stress* oxidativo, a atividade da glutationa S-transferase (GST) não se alterou com a contaminação, mas o aquecimento provocou um aumento na atividade de GST. Sob contaminação, a GST apresentou níveis mais elevados nos organismos provenientes do tratamento controlo (temperatura e pH) quando comparado com os organismos do tratamento de aquecimento e hipercapnia. A atividade da catalase não apresentou diferenças significativas nos organismos dos tratamentos não contaminados, mas com a contaminação a enzima aumentou nos peixes sujeitos à temperatura controlo e hipercapnia. Por último, a atividade da superóxido dismutase (SOD) não apresentou diferenças nos peixes dos tratamentos não contaminados, mas apresentando níveis mais elevados nos organismos sujeitos a contaminação no tratamento de temperatura controlo e hipercapnia.

Este estudo parece indicar que a corvina conseguirá adaptar-se às condições climáticas expectáveis no futuro em ambientes contaminados com MeHg. O aquecimento será o fator que mais afetará a acumulação de mercúrio na corvina e algumas variáveis irão ser afetadas pela contaminação. Embora a corvina tenha sofrido algumas alterações a nível fisiológico, estas não foram nocivas para o peixe. Em conclusão, estes resultados indicam, que a corvina poderá adaptar-se às futuras condições climáticas mesmo sob contaminação de mercúrio.

Palavras-chave:

Corvina, alterações climáticas, mercúrio, bioacumulação, encefalização, neurotransmissão colinérgica, *stress* oxidativo.

Abstract

The increase in anthropogenic carbon dioxide (CO_2) in the atmosphere is leading to higher ocean temperature and lower pH. At the same time, mercury released into the environment is also a fact, being deposited in the ocean. Thus, marine animals are being challenged by ocean warming, acidification and contamination simultaneously. However, their biological and physiological responses to these environmental changes still remain unknown. This study evaluated meagre (*Argyrosomus regius*) responses to the effects of ocean warming (+4ºC), acidification (ΔpH = 0.5 units) and MeHg exposure (8.02 mg kg^{-1} dw), on Fulton condition index, degree of encephalization, acetylcholinesterase (AChE) levels, heat shock response (HSR), lipid peroxidation [malondialdehyde (MDA) concentration] and antioxidant enzyme activities [glutathione S-transferase (GST), catalase and superoxide dismutase (SOD)]. Temperature was the factor leading to an increase of MeHg accumulation in meagre. Survival and Fulton condition were not affected by any of the factors. Encephalizaton coefficient was only affected under the warming and hypercapnia non-contaminated treatment and only brain stem increased in response to warming. Moreover, AChE was only influenced under contaminated treatments. HSR only varied in muscle tissue and was mostly affected by warming treatments (non-contaminated and contaminated). In contrast, MDA levels were not influenced by warming, hypercapnia or contamination. GST increased under the warming non-contaminated treatment. Catalase was higher in samples from the contaminated hypercapnia treatment compared to the corresponding non-contaminated treatment, whereas SOD reached higher levels under the hypercapnia contaminated treatment. The present study shows that meagre will not suffer major consequences under predicted future ocean contaminated conditions. Although meagre suffered physiological alterations under these conditions, these responses were not harmful for the fish. These results suggest that meagre will be able to adapt to the predicted future ocean conditions even under mercury contamination. However, future studies will be necessary to comprehensively understand how marine biota will respond to the combined effects of future warming, acidification and pollution

Keywords:

Meagre, climate change, mercury, bioaccumulation, encephalization, cholinergic neurotransmission, oxidative stress

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1. Introduction

1.1. Global warming

In the preindustrial era the atmospheric carbon dioxide (CO_2) concentrations were approximately 280 ppm (Caldeira and Wickett, 2003; IPCC, 2013) and are now exceeding 390 ppm (IPCC, 2013). By the year 2100, it is expected that atmospheric CO_2 concentrations reach values between 420 and 940 ppm, depending on the emissions scenario (IPCC, 2014). This increase in CO_2 (and other greenhouse gases) is causing a rise in temperature and therefore global warming (IPCC, 2013) (Fig. 1). Researchers predict a temperature rise between 0.3 °C to 4.8 °C by the year 2100 (IPCC, 2013). Sea surface temperature (SST) has significantly warmed during the past 30 years and it is predicted that will continue to increase, especially in coastlines (IPCC, 2014). Warming affects the performance of marine organisms at different stages in their life history through changes in physiology, morphology and behavior (Harley et al., 2006). Species with ample thermal windows, short generation times and a range of genotypes in populations will be favored under warming conditions (Pörtner and Farrell, 2008). Ultimately, species thermal tolerance and adaptability will determine the distribution and abundance of species under these conditions (Harley et al., 2006).

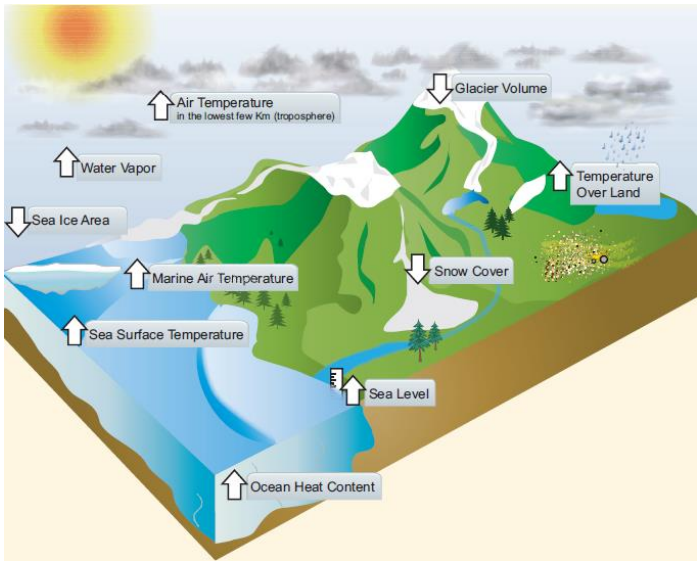


Figure 1. Components of the climate system that would be expected to change in a warming world exhibit trends consistent with warming (arrow direction denotes the sign of the change) (IPCC, 2013).

1.2 Ocean acidification

Simultaneously, oceans are absorbing CO_2, changing their chemical balance (IPCC, 2013). Dissolved CO_2 increases the bicarbonate ion (HCO_3^-) and causes a decrease in carbonate ion (CO_3^{2-}) (IPCC, 2013). Ocean CO_2 uptake causes a decrease in ocean pH, resulting in ocean acidification (IPCC, 2013). Since the preindustrial time, ocean surface water pH has decreased 0.1 units and it is expected to decrease up to 0.13-0.42 units until 2100 (IPCC, 2014), however these values may be higher in coastal areas (IPCC, 2014). Ocean acidification will have impacts especially on calcifying organisms due to the reduction in seawater pH and carbonate ion concentrations which causes calcium carbonate ($CaCO_3$) saturation (Azevedo et al., 2015; Hoegh-Guldberg et al., 2007; Orr et al., 2005) (Fig 2). The reduction of carbonate-ions concentrations diminishes the rate of calcification of marine organisms weakening carbonate skeletal structures (Raven et al., 2005). Besides calcifying species, ocean acidification will also have an impact in other organisms, such as fish (Ishimatsu et al., 2008). High pCO_2 may involve an extra energy expenditure for physiological adaptations, especially in acid-base regulation (Ishimatsu et al., 2008). It has also been demonstrated that hypercapnia causes alterations in fish biological responses like aerobic scope (Munday et al., 2009; Rummer et al., 2013), decreases growth and thermal tolerance (Checkley et al., 2009; Pimentel et al., 2014b) and behavioral changes (Cripps et al., 2011; Munday et al., 2010; Pimentel et al., 2014a).

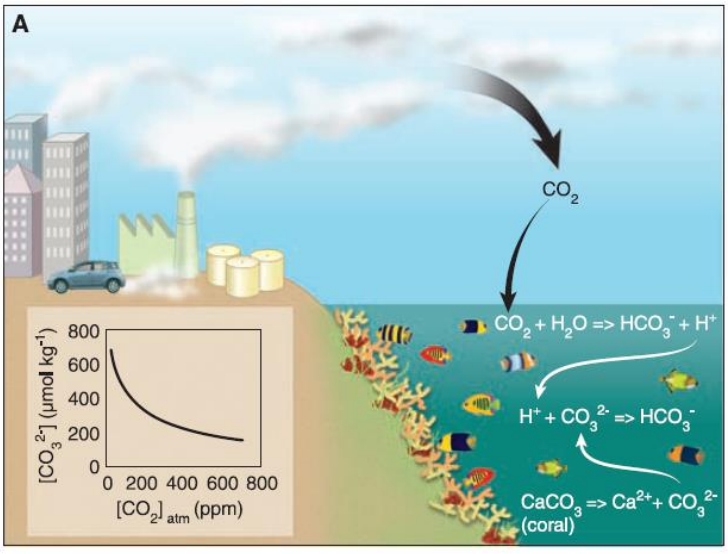


Figure 2. Linkages between the buildup of atmospheric CO_2 and the slowing of coral calcification due to ocean acidification (Hoegh-Guldberg et al., 2007).

These global ocean changes are expected to cause negative responses in marine organisms, essentially because of the synergistic effects between these two factors.

These effects have already been reported by various studies in diverse marine biota. Investigators demonstrated that in marine organisms, the synergism between warming and acidification has a higher negative effect on calcification, survival, and reproduction than when stressors act in isolation (Harvey et al., 2013). Predicted ocean warming and acidification caused shorter embryonic periods, lower survival rates and enhanced premature hatching in the cuttlefish and have drastic effects in survival rates of summer embryos of a coastal squid (Rosa et al., 2014b; Rosa et al., 2013). Pimentel et al., (2015) demonstrated that the effects on several physiological and biochemical variables in flatfish larvae change greatly under both ocean warming and acidification. Munday et al., (2009) also reported that aerobic scope and mortality in reef fish are affected by ocean warming and acidification. Pörtner and Farrell, (2008) presented a conceptual model of how synergistic stressors like ocean acidification (by CO_2) narrow thermal windows of aerobic performance according to species-specific sensitivities (Fig. 3).

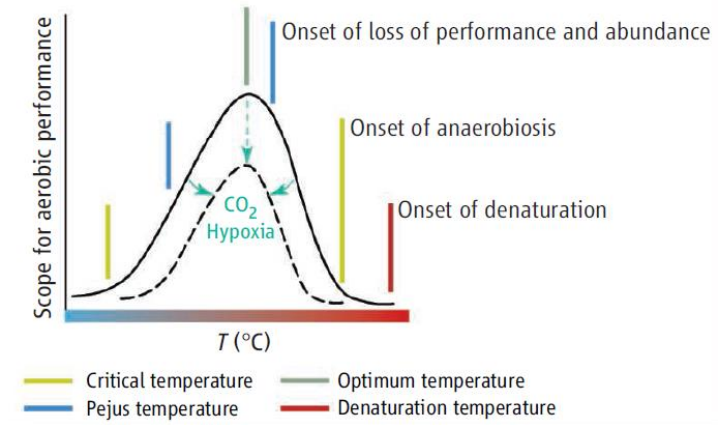


Figure 3. Temperature effects on the thermal windows of aerobic performance on aquatic animals. Synergistic stressors like ocean acidification (by CO_2) narrow thermal windows (Pörtner and Farrell, 2008).

1.3 Mercury contamination

In addition to global warming and ocean acidification, marine biota is also dealing with another stress factor: pollution. Marine ecosystems are becoming contaminated with several toxic compounds including metals. One of these metals is mercury, which is a very toxic element, as well as its compounds (Korbas et al., 2011). Mercury releases into the environment can occur through natural and anthropogenic sources (e.g. coal combustion, industrial activities, mining) (Nriagu and Pacyna, 1988; Richter et al., 2014). After the industrial revolution, mercury anthropogenic emissions increased drastically (Sunderland and Mason, 2007) and predictions say it is likely they will continue to increase (Streets et al., 2009). Nowadays mercury emissions are mostly of

anthropogenic origin and part of these emissions are deposited in the ocean sediment (Selin et al., 2008; Sunderland and Mason, 2007) (Fig. 4).

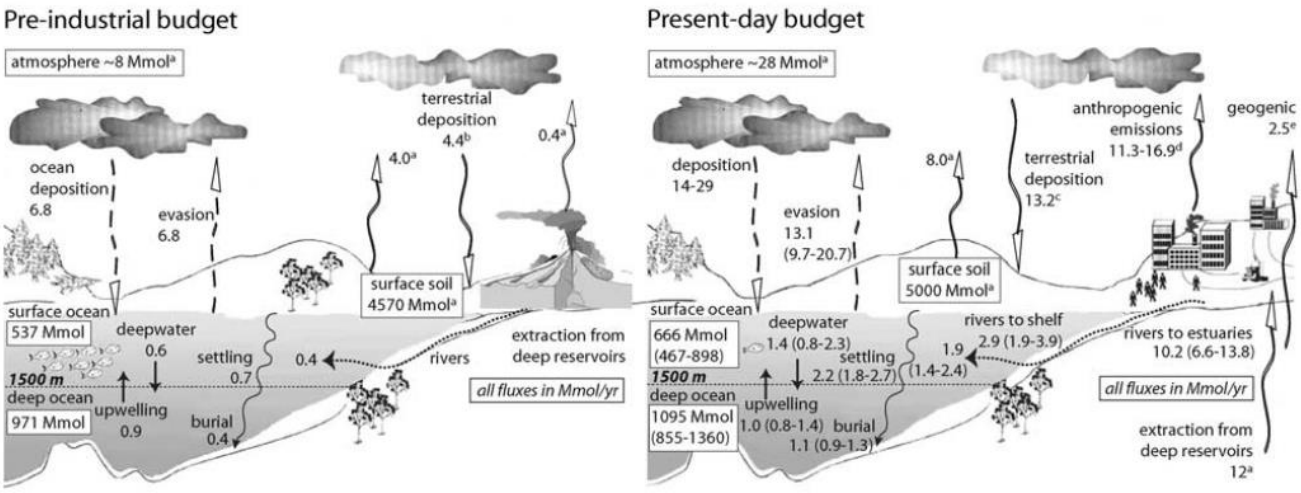


Figure 4. Global budgets for preindustrial and current mercury cycling in oceans (Sunderland and Mason, 2007).

Bacteria present in aquatic sediments transform the inorganic mercury form into methylmercury (MeHg), which is highly toxic (Richter et al., 2014). Due to the biochemical conditions that lead the Hg transformation in MeHg by bacteria, estuaries and coastal areas are favorable zones for this transformation to occur (Dijkstra et al., 2013). This compound bioaccumulates in aquatic organisms and goes through biomagnification in aquatic food webs, increasing its toxicity and reaching elevated concentrations in high trophic level biota (Campbell et al., 2005; Evers et al., 2011). Hence fish consumers, including humans, are at high risk of exposure to MeHg (Richter et al., 2014). MeHg comprises around 90–95% of the total mercury (HgT) in fish (Burger et al., 2003; Gray et al., 2000). It can cause several adverse effects in these animals, for example, disturb reproductive performance (Hammerschmidt et al., 2002; Matta et al., 2001), enhance oxidative stress (Berntssen et al., 2003; Gonzalez et al., 2005; Keyvanshokooh et al., 2009; Larose et al., 2008; Mieiro et al., 2010), alter activity of enzymes of the central nervous systems (De Domenico et al., 2013; Gill et al., 1990) and change swimming behavior and predator avoidance (Weis and Weis, 1995; Zhou and Weis, 1998).

1.4 Synergisms

Although marine organisms will have to deal with the effects of ocean warming, acidification and contamination simultaneously, little is known about the interactions between climate change and contaminants on marine organisms. The impacts of climate

change on marine species can be aggravated by pollution (Noyes et al., 2009). Interactions between temperature and metal pollution influence the physiological tolerance to both stress factors in aquatic ectotherms (Sokolova and Lannig, 2008). Methylmercury bioaccumulation will increase at lower trophic levels under the predicted temperature rise, which consequently will increase MeHg levels throughout the food chain (Dijkstra et al., 2013). Elevated temperature exacerbates biological responses in freshwater fish exposed to metal contamination (Dorts et al., 2014; Lapointe et al., 2011; Sappal et al., 2014). Moreover, ocean acidification can also intensify metal toxicity (Han et al., 2014). For instance, it has been shown that acidification enhances the toxicity of copper in the polychaete *Arenicola marina*, showing that there are higher toxicity responses under reduced seawater pH (Campbell et al., 2014). Furthermore, temperature and pH affect the bioaccumulation of different metals in cephalopods early life stages (Lacoue-Labarthe et al., 2012; Lacoue-Labarthe et al., 2009; Lacoue-Labarthe et al., 2011).

Only few studies were conducted regarding climate change and contamination responses of marine organisms and, to my knowledge, none uses mercury as the metal contaminant. Most of them just evaluate the bioaccumulation in the organisms or visible responses such as mortality. Also, in fish, this type of response is usually analyzed in freshwater species. That said, there is a lack of knowledge in the response of marine biota, particularly in fish, to ocean warming, acidification and contamination simultaneously.

1.5 Stress biomarkers

As previously said, climate change can cause differences in the accumulation of mercury in fish (Dijkstra et al., 2013) plus, mercury has a great effect in the nervous system (Baatrup, 1991). In teleost fish, brain grows continuously during its lifetime (Ekström, 1994; Kotrschal et al., 1998) and can be affected by environmental conditions such as habitat complexity or different rearing conditions especially in early stages (Kihslinger and Nevitt, 2006; Pollen et al., 2007). Acetylcholinesterase (AChE) is an important enzyme in the nervous system because it hydrolyses the neurotransmitter acetylcholine (Romani et al., 2003). It has been reported that near-future carbon dioxide can alter neurotransmitter function in fish (Nilsson et al., 2012) and it is known that this enzyme is affected by mercury (Gill et al., 1990; Richetti et al., 2011). Nonetheless, no information exists on how ocean warming, acidification and mercury contamination will influence AChE response in fish.

Heat shock proteins (HSP) production occurs in response to high temperatures (Repolho et al., 2014; Rosa et al., 2014a; Rosa et al., 2012) and metal contamination

(Rajeshkumar and Munuswamy, 2011; Williams et al., 1996). High temperatures cause formation of oxygen reactive species (ROS) (Abele et al., 2002; Lesser, 2006) and HSP production helps repairing, refolding and eliminating damaged or denatured proteins and protect and control ROS formation (Sokolova et al., 2011). The increase in ROS may also cause lipid peroxidation, one of the most common cellular injury mechanisms (Lesser, 2006). ROS production induce antioxidant enzymes activity, such as superoxide dismutase (SOD), which converts superoxide (O_2^-) into hydrogen peroxide (H_2O_2); catalase (CAT) which converts H_2O_2 into water (H_2O) and oxygen (O_2) and glutathione S-transferase (GST), which is involved in the protection against xenobiotics (Lesser, 2006; Wang et al., 2000).

2. Objectives

The aim of this study was to evaluate the biological responses of juvenile meagre (*Argyrosomus regius*), to the effects of ocean warming (+4ºC), acidification (ΔpH = 0.5 units) and MeHg exposure (8.02 mg kg^{-1} dw). More specifically, I determined the combined impact of such climate-related variables and contamination on Fulton condition index, degree of encephalization, acetylcholinesterase levels, heat shock response, lipid peroxidation and antioxidant enzyme activities. It is worth noting that *A. regius* is a resilient fish and can easily adapt to changes in the environment (Monfort, 2010). However, this species is found in coastlines and estuaries, especially in the juvenile stage, where climate change and mercury will have more impact in the future. Meagre is a high commercial value fish (El-Shebly et al., 2007), thus being important to evaluate the future effects of these stressors on this species.

3. Material and methods

3.1. Collection of specimen and incubation

Juvenile organisms of *Argyrosomus regius* (n = 85; Fig. 5) (mean ± standard deviation; total weight: 4.26 ± 2.8 g; total length: 6.30 ± 1.2 cm), from IPMA's aquaculture pilot station (EPPO, Olhão, Portugal) were transported to the aquaculture facilities of Laboratório Marítimo da Guia (LMG, MARE, Faculty of Sciences) in August 2014. The organisms were randomly placed in eight 90L tanks with separate recirculating aquaculture systems (RAS) filled with filtered natural seawater and equipped with glass wool, bio-balls and protein skimmers to maintain water quality.

Figure 5. Meagre (*Argyrosomus regius*).

After an acclimation period of 15 days, fish were subjected to ocean warming (+4 °C) and acidification (ΔpH = 0.5 units) and MeHg contamination conditions, more specifically, the trial was divided in eight treatments: i) 19 °C, pH 8.0 (control conditions) and control feed (MeHg: 0.06 mg kg^{-1} dw; HgT: 0.07 mg kg^{-1} dw), ii) 19 °C, pH 8.0 and contaminated feed (MeHg: 8.02 mg kg^{-1} dw; HgT: 8.28 mg kg^{-1} dw), iii) 19 °C, pH 7.5 (control temperature and hypercapnic scenario) and control feed (MeHg: 0.06 mg kg^{-1} dw; HgT: 0.07 mg kg^{-1} dw), iv) 19 °C, pH 7.5 and contaminated feed (MeHg: 8.02 mg kg^{-1} dw; HgT: 8.28 mg kg^{-1} dw); v) 23 °C, pH 8.0 (warming scenario with control pH) and control feed (MeHg: 0.06 mg kg^{-1} dw; HgT: 0.07 mg kg^{-1} dw); vi) 23 °C, pH 8.0 and contaminated feed (MeHg: 8.02 mg kg^{-1} dw; HgT: 8.28 mg kg^{-1} dw); vii) 23 °C, pH 7.5 (warming and hypercapnic scenario) and control feed (MeHg: 0.06 mg kg^{-1} dw; HgT: 0.07 mg kg^{-1} dw); and vii) 23 °C, pH 7.5 and contaminated feed (MeHg: 8.02 mg kg^{-1} dw; HgT: 8.28 mg kg^{-1} dw), with ten to twelve fish per treatment, during 30 days. The MeHg exposure occurred through the feed. Fish were fed two to three times a day and the

quantity of food provided was approximately 1% of animal weight per day. Salinity was kept at 35.0 ± 1.0 g/L and the photoperiod was fixed to 12 h light: 12 h dark. Temperature in the tanks was controlled and maintained using chillers, and pH was maintained with a Profilux system. Temperature, pH and salinity were measured every day. Ammonia and nitrite were regularly monitored and kept within recommended levels. Seawater carbonate system speciation (Table S1) was calculated once a week from total alkalinity (determined according to Sarazin et al., (1999) and pH measurements). Total dissolved inorganic carbon (CT), pCO_2 and aragonite saturation were calculated using the CO2SYS software (Lewis et al., 1998), with dissociation constants from Mehrbach et al., (1973) as refitted by Dickson and Millero, (1987).

3.2. Diet

The non-contaminated and contaminated fish were fed with different diets. The composition of the two diets (Table 1) was similar diverging in one aspect: the contaminated diet was fortified with MeHg (inserted in the form of MeHg(II) chloride, CH_3ClHg, 99.8 %, Sigma-Aldrich, previously solubilized in ethanol). The pellet given to the fish in the contaminated treatment had approximately 8.02 mg kg^{-1} dw of MeHg and 8.28 mg kg^{-1} dw of HgT (Table 2).

Table 1. Composition of the two diets (i.e. dry inert pellets) used for juvenile meagre feeding.

Ingredients	**%**
Fishmeal LT70	28
Fishmeal 60	20
Fish soluble concentrate	2.5
Soy protein concentrate	5
Wheat gluten	5.5
Maize gluten	5
Soybean meal 48	9
Wheat meal	5.5
Whole peas	5
Fish oil	13.5
Vitamin and mineral premix	1
Dry matter (DM), %	94.3
Crude protein, %DM	51.7

Crude fat, %DM	18.9
Ash, %DM	9.6

Table 2. Total mercury and methylmercury concentrations in the non-contaminated and contaminates feed

Feed	HgT (mg kg^{-1})	MeHg (mg kg^{-1})
Non-contaminated	0.07 ± 0.00	0.06 ± 0.00
Contaminated	8.28 ± 0.10	8.02 ± 0.10

3.3. Total mercury and methylmercury accumulation

Methylmercury was extracted from the samples (fish and feeds) as described by Scerbo and Barghigiani, (1998), i.e. freeze-dried samples (approximately, 200 mg) were hydrolyzed in 10 ml of hydrobromic acid (47 % w/w, Merck), followed by MeHg extraction with 35 mL toluene (99.8 % w/w, Merck) and toluene removal with 6 ml cysteine aqueous solution (1 % L-cysteinium chloride in 12.5 % anhydrous sodium sulfate and 0.775 % sodium acetate; Merck). Then, total Hg and MeHg were determined in all samples (10-15 mg for solids or 100-200 µL for liquids) by atomic absorption spectrometry (AAS), following the method 7473 of the EPA, (2007), using an automatic Hg analyser (AMA 254, LECO, USA). Mercury concentrations were calculated from linear calibration (using, at least, five different standard concentrations), with a Hg(II) nitrate standard solution (1000 mg L^{-1}, Merck) dissolved in nitric acid (0.5 mol L^{-1}, Merck), and the detection limit was 0.005 mg kg^{-1}, wet weight (ww). Accuracy was checked through the analysis of the certified reference material DORM-4, and results obtained in the present study were within the certified range of values (Table 3). A minimum of three measurements (replicates) were performed per sample. Blanks were always tested in the same conditions as the samples. Prior to utilization, all laboratory ware was cleaned with nitric acid (20 % v/v) for 24h and rinsed with ultrapure water to avoid contamination. All standards and reagents were of analytical (pro analysi) or superior grade.

Table 3. Laboratory performance on standard reference materials for total mercury and methylmercury (mg kg^{-1} dry weight).

	Standard reference material	HgT	MeHg
Present work	DORM-4*	0.390 ± 0.025	0.354 ± 0.031
Certified value		0.410 ± 0.055	0.353 ± 0.062

*fish protein certified reference material for trace metals, National Research Council Canada, Canada

3.4. Survival and Fulton condition

Survival rate was calculated as the percentage of live fish the end of the experiment, with respect to the number of fish at the beginning. After being euthanized fish were weighed and measured (TL). Fulton's condition (measure of individual fish's health) was calculated using the formula $K = (weight/TL^3) \times 100$.

3.5. Brain sampling

Fish were euthanized with an overdose of tricaine mesylate solution (MS222, Pharmaq; 500–1000 mg/L) and the spinal cord sectioned to be sure that the animal was dead. The brain was then visually macro-dissected from the cranial cavity, under a stereoscope. Afterwards, brain was dissected into five macro areas: telencephalon, diencephalon, optic tectum, cerebellum and brain stem (Fig. 6) and each area was weighted in an analytical Sartorius balance.

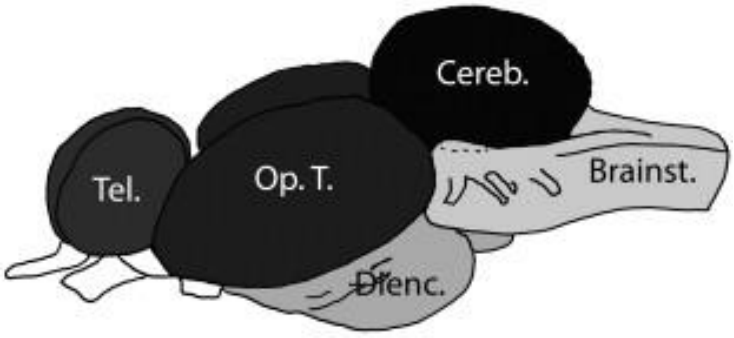


Figure 6. Fish brain macro areas: telencephalon, optic tectum, cerebellum, diencephalon and brainstem (Gonçalves et al., 2008)

3.6. Acetylcholinesterase (AChE) activity

Meagre brain areas were homogenized on ice in 1.0 mL of PBS solution (0.14M NaCl, 253 0.003M KCl, 0.01M Na2HPO4, 0.002M KH2PO4, pH 7.4) using a teflon tissue grinder for microtubes. The crude homogenates were then centrifuged (10 min at 14,000 x g at 4 °C). Then, 100 µl of supernatant were diluted to 1:2 in sodium phosphate buffer (50 mM, pH 8). The determination of acetylcholinesterase activity (EC 3.1.1.7; AChE) was performed based on an optimized Ellman method adapted to 96-well microplate (Ellman et al., 1961; Magnotti et al., 1987), in which thiocholine produced by the action of acetylcholinesterase forms a yellow (nitrobenzoate) color with 5,5'-dithiobis(2-nitrobenzoic acid), proportional to the AChE activity. All chemicals used in the assay were purchased to Sigma-Aldrich (Steinheim, Germany). Briefly, 50 µL of diluted samples, were added to each 96 microplate well (Greiner, Germany). Additionally, 50 µL of assay buffer were added to two microplate wells for blanks. Then an acetylthiocholine reaction mixture was prepared freshly in ultra-pure water [50 mM phosphate buffer; 75 mM Acetylthiocholine iodide solution; 1.0 mM of 5,5'-dithiobis(2-nitrobenzoic acid)] and 250 µL were added to each microplate well. Afterwards, the microplate was gently shaken for 30s at room temperature and absorbance read at 415 nm in a microplate reader (Bio-Rad, Benchmark model, USA) at every minute during 10 minutes. Then, AChE activity was calculated considering that one unit of enzyme catalyzes the production of 1.0 µmole of thiocholine per minute, under the assay conditions, and expressed as nmol min^{-1} mg^{-1} of total protein following normalization.

3.7. Heat shock response, lipid peroxidation and antioxidant enzyme activities

3.7.1. Preparation of tissue extracts:

Muscle, liver and gills samples (n = 6 per treatment) were homogenized (Ultra-Turrax, Ika, Staufen, Germany) in accordance to body mass of each sample in homogenization buffer, 300 mg tissue per 1 ml phosphate buffered saline solution (PBS, pH 7.3): 0.14 M NaCl, 2.7 mM KCl, 8.1 mM Na_2HP0_4, 1.47 mM KH_2P0_4] by using a glass/PTFE Potter Elvehjem tissue grinder (Kartell, Turin, Italy). All homogenates were then centrifuged (20 min at 14 000 g at 4 °C) and the antioxidant enzyme activities, lipid peroxidation and heat shock response (HSR) were quantified in the supernatant fraction. All enzyme assays were tested with commercial enzymes obtained from Sigma-Aldrich (St. Louis, MO, USA), and each sample was run in triplicate. The enzyme results were

normalize by measuring the total protein content of the samples according to the Bradford method (Bradford, 1976).

3.7.2. Heat shock proteins

Heat shock protein (HSP70/HSC70) content was assessed by Enzyme-Linked Immunoabsorbent Assay (ELISA) adapting a protocol from Njemini et al., (2005). Briefly, 10 µl of the homogenate's supernatant was diluted in 250 µl of PBS (1x), and 50 µl of the diluted sample was added to a 96-well microplates (Nunc brand, Roskilde, Denmark) and allowed to incubate overnight at 4 °C. In the next day, the microplates were washed (3X) in 0.05% PBS-Tween-20. One hundred microliters of blocking solution [1% bovine serum albumin (BSA) Sigma-Aldrich] was added to each well and left to incubate at room temperature for 2 h. After washing the 96-well plates, 50 µl of 5 µg ml^{-1} primary antibody (anti-HSP70/HSC70, Acris, San Diego, CA, USA), detecting 72 and 73 kDa proteins corresponding to the molecular mass of inducible and constitutive isoforms, respectively, was added to each well and then incubated overnight at 4 °C. According to the manufacturer details the primary antibody HSP70/HSC70 (AM12032PU-N) has a broad range reactivity including diverse fish species. On the next day, the nonlinked antibody was removed by washing the microplates again, which were then incubated for 2 h at room temperature with 50 µl of 1 µg ml^{-1} of the secondary antibody, antimouse IgC, Fab specific, alkaline phosphatase conjugate (Sigma-Aldrich). After three additional washes, 100 µl of substrate (SIGMA FASTTM p-Nitrophenyl Phosphate Tablets, Sigma-Aldrich) was added to each well and incubated 10-30 min at room temperature. Stop solution (50 µl; 3 N NaOH) was added to each well, and the absorbance was then read at 405 nm in a 96-well microplate reader (Asys UVM 340, Biochrom, USA). The amount of HSP70/HSC70 present in the samples was calculated from a curve of absorbance based on serial dilutions of purified HSP70 active protein (Acris) to give a range from 0 to 2000 ng ml^{-1}. The results were expressed in relation to the sample total protein (ng mg^{-1}total protein).

3.7.3. Lipid peroxides assay (malondialdehyde concentration)

Lipid peroxides assay was determined by the quantification of a specific end-product of the oxidative degradation process of lipids, malondialdehyde (MDA). The thiobarbituric acid reactive substances (TBARS) protocol (Uchiyama and Mihara, 1978) was used. Briefly, 5 µl of each sample, treated as described before, were added to 45 µl of 50 mM monobasic sodium phosphate buffer, followed by adding 12.5 µl of sodium dodecyl sulfate (8.1%), 93.5 µl of trichloroacetic acid (20%, pH = 3.5) and 93.5 µl of

thiobarbituric acid (1%) to each microtube. Then, 50.5 µl of ultrapure water was added to this mixture and placed in a vortex for 30 s. Afterward, a needle was used to puncture the lids and microtubes were incubated in boiling water (10 min) followed by cooling on ice. Subsequently, 62.5 µl of ultrapure water and 312.5 µl of *n*-butanol pyridine (15 : 1, v/v) (Sigma-Aldrich, Hamburg, Germany) were added and microtubes centrifuged (5000 x g; 5 min.). Triplicates of 150 µl of the supernatant of each reaction were put into a 96-well microplate and absorbance was read at 532 nm. To quantify the lipid peroxides (MDA content), an eight-point calibration curve (0–0.3 µM TBARS) was calculated using malondialdehyde (dimethylacetal) (MDA) (Merck, Switzerland) standards. Results were expressed in relation to the sample total protein (nmol mg^{-1} total protein).

3.7.4. Glutathione S-Transferase (GST) activity

Glutathione S-transferase (GST) total activity (EC 2.5.1.18) was determined according to the procedure described by Habig et al., (1974) and optimized for 96-well microplate (Sigma Technical Bulletin, CS0410). This assay uses 1-Chloro-2,4-dinitrobenzene (CDNB) as substrate and, upon conjugation of the thiol group of glutathione to the CDNB substrate, there is an increase in the absorbance. Therefore, the enzyme activity was determined spectrophotometrically by measuring the formation of the conjugates of glutathione (GSH) and l-chloro-2,4-dinitrobenzene (CDNB). The assay contained 200 mM L-glutathione (reduced), Dulbecco's PBS and 100 mM 1-chloro-2,4-dinitrobenzene (CDNB) solution. Equine liver GST (Sigma-Aldrich) was used as positive control to validate the assay. Then, to perform the assay, 180 µl of substrate solution were added to 20 µl of GST standard or sample in each well of a 96-well microplate (Nunc-Roskilde) and the absorbance at 340 nm was recorded every minute for 6 min, using a plate reader (Asys UVM 340, Biochrom, USA). Thereby the increase in absorbance is directly proportional to GST activity. Then, the GST activity was calculated using a molar extinction coefficient for CDNB of 5.3 εmM (Sigma Technical Bulletin, CS0410), as follows:

$$GST\ activity = \frac{\Delta A_{340}/min}{0.0053} \times \frac{Total\ volume}{Sample\ volume} \times dilution\ factor$$

The result are expressed in relation to total protein of the sample (nmol min^{-1} mg^{-1} total protein)

3.7.5. Catalase (CAT) activity

Catalase activity was assessed through and adaptation of the method described by Johansson and Borg, (1988). In this assay, 20 µl of each sample, 100 µl of 100 mM potassium phosphate and 30 µl of methanol were added to a 96-well microplate, which was promptly shaken and incubated for 20 minutes. Afterwards, 30 µl of potassium hydroxide (10 M KOH) and 30 µl of purpald (34.2 mM in 0.5 M HCl) were added to each well, and the plate shaken and incubated for another 10 minutes. Subsequently, 10 µl of potassium per iodate (65.2 mM in 0.5 M KOH) was added to each well and a final incubation was performed for 5 minutes. Using a microplate reader (BIO-RAD, Benchmark, USA), enzymatic activity was determined spectrophotometrically at 540 nm. Formaldehyde concentration of the samples was calculated based on a calibration curve (from 0 to 75 µM formaldehyde), followed by the calculation of the CAT activity of each sample, where one unit of catalase is defined as the amount that will cause the formation of 1.0 nmol of formaldehyde per minute at 25 ºC. The results are expressed in relation to total protein content (nmol min^{-1} mg^{-1}protein).

3.7.6. Superoxide dismutase (SOD) activity

The SOD assay follows the nitro blue tetrazolium (NBT) method adapted from Sun et al., (1988). In this method, superoxide radicals (O_2^-) are generated by the reaction of xanthine with xanthine-oxidase (XOD), and reduce NBT to formazan. SOD competes with NBT for the dismutation of O_2^- into peroxide (H_2O_2) and molecular oxygen. The assay was performed using a 96-well microplate (Nunc-Roskilde), adding to each well 200 µl of 50 mM phosphate buffer (pH 8.0) (Sigma-Aldrich), 10 µl of 3 mM EDTA (Riedel-de Haën, Seelze, Germany), 10 µl of 3 mM xanthine (Sigma-Aldrich), 10 µl of 0.75 mM NBT (Sigma-Aldrich) and 10 µl of SOD standard or sample. The reaction started with the addition of 100 mU XOD (Sigma-Aldrich) and the absorbance at 560 nm was recorded every minute for 5 minutes, using a plate reader (Asys UVM 340, Biochrom, USA) at 25 °C. A negative control included all components except SOD or sample, producing a maximal increase in absorbance at 560 nm, which allowed determining the inhibition percentage per minute, caused by SOD activity. SOD from bovine erythrocytes (Sigma-Aldrich) was used as standard and positive control. The SOD inhibition percentage was expressed in % inhibition mg^{-1} of total protein.

3.8. Statistical analyses

Three-way ANOVAs (repeated measurements; using pH and temperatures and contamination) were conducted to detect significant differences in mercury accumulation, Fulton condition, acetylcholinesterase activity, heat shock response, lipid peroxidation and antioxidant enzyme activities. The differences in encephalization coefficient and macro areas coefficient were investigated in the following manner: for the encephalization coefficient a linear regression was made with the brain masses values of non-contaminated control conditions (19 °C pH 8.0), with these linear regression, predicted brain mass was calculated for the respectively fish mass of each fish of each condition. For the macro area coefficient five linear regressions were made with each brain macro area of non-contaminated control conditions (19 °C pH 8.0) and with these linear regressions, predicted macro areas masses were calculated for the respectively total brain mass of each fish and condition. Residuals were calculated with predicted mass value minus the observed mass and outliers were excluded using the ROUT method. Then, three-way ANOVAs (with temperature, acidification and contamination as variables) were conducted to detect significant differences in all variables measured. All statistical analyses were performed for a significance level of 0.05, using Statistica 12 software (StatSoft Inc., Tulsa, OK, USA).

4. Results

4.1. Total mercury accumulation

At the end of the 30-days trial, the warming and control pH condition elicited the highest mercury accumulation in most tissues analyzed (Fig. 7; $P < 0.05$). Under control conditions, gills and liver revealed higher levels of accumulation (6.25 mg kg^{-1} and 7.06 mg kg^{-1}, respectively) than muscle (3.74 mg kg^{-1}) ($P < 0.05$). In contrast, under the control temperature and hypercapnia treatment (19 °C, pH 7.5), muscle accumulated less mercury (4.57 mg kg^{-1}) than liver (9.84 mg kg^{-1}) and the same happened under the warming and control pH condition ($P < 0.05$). Under the warming and hypercapnia scenario (23 °C, pH 7.5) gills accumulated less mercury (3.25 mg kg^{-1}) than brain (5.29 mg kg^{-1}) ($P < 0.05$).

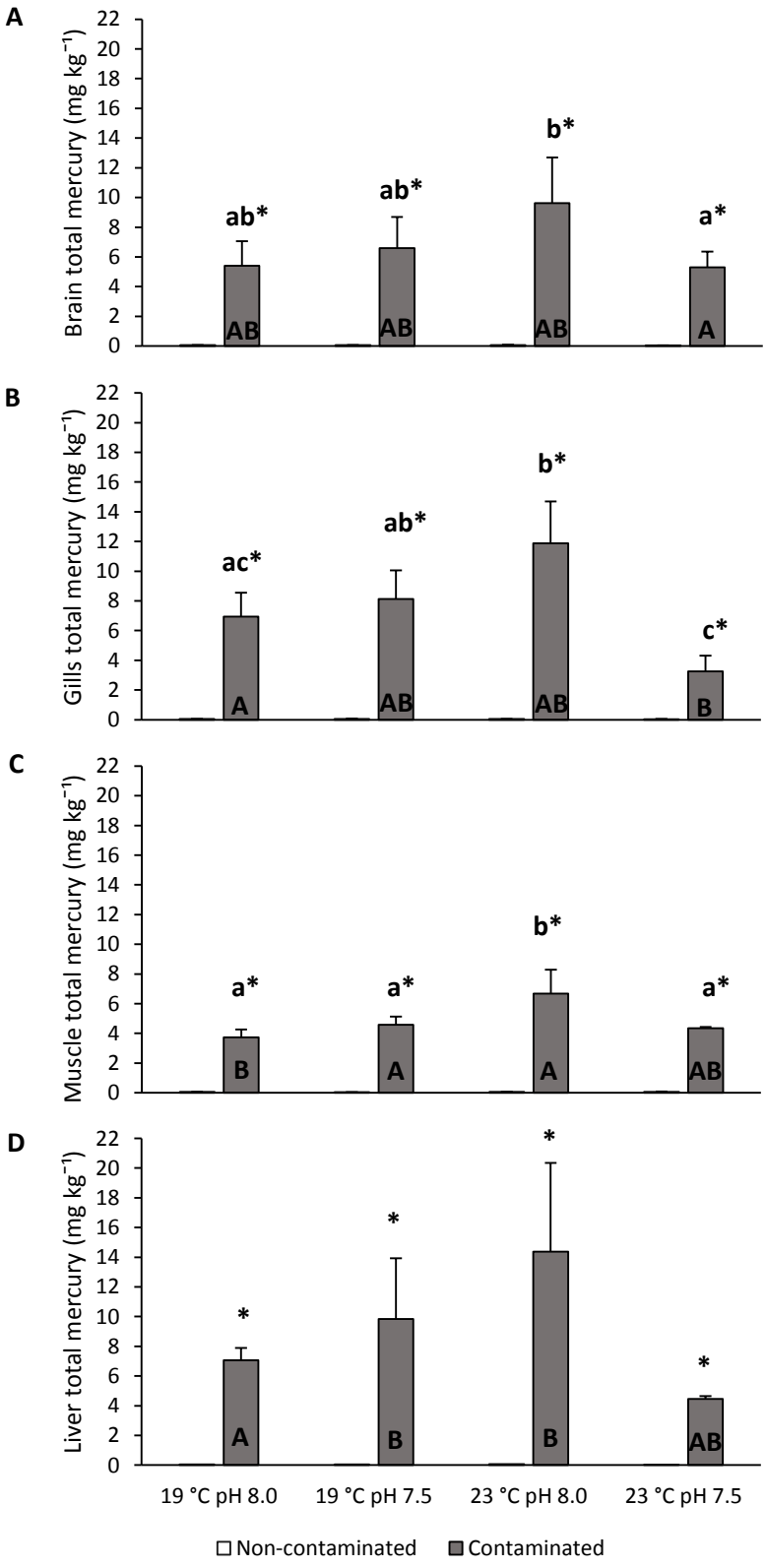


Figure 7. Impact of ocean acidification (ΔpH 0.5) and warming (+4 °C) on total mercury accumulation on A) brain B) gills, C) muscle and D) liver of juvenile meagre (*Argyrosomus regius*) exposed to methylmercury for 30 days. Values represent mean+s.d. Small letters represent statistical differences among treatments in the same contamination condition. Asterisk represents significant differences between contaminated and non-contaminated treatments in each temperature and pH condition. Capital letters represent significant differences between tissues in the same treatment.

4.2. Survival, Fulton condition and encephalization coefficient

During the trial, no mortalities were registered in all treatments. Furthermore, Fulton condition (*K*) did not show any significant differences between treatments (Fig. 8A; $P > 0.05$). Warming and hypercapnia changed significantly the encephalization coefficient under the non-contaminated condition (Fig. 8B; $P < 0.05$). Under this condition, brain was significantly bigger than its predicted value. Under contaminated conditions, encephalization coefficient did not displayed significant differences ($P > 0.05$), however, it was significantly different between the non-contaminated and contaminated warming and hypercapnia scenarios ($P < 0.05$).

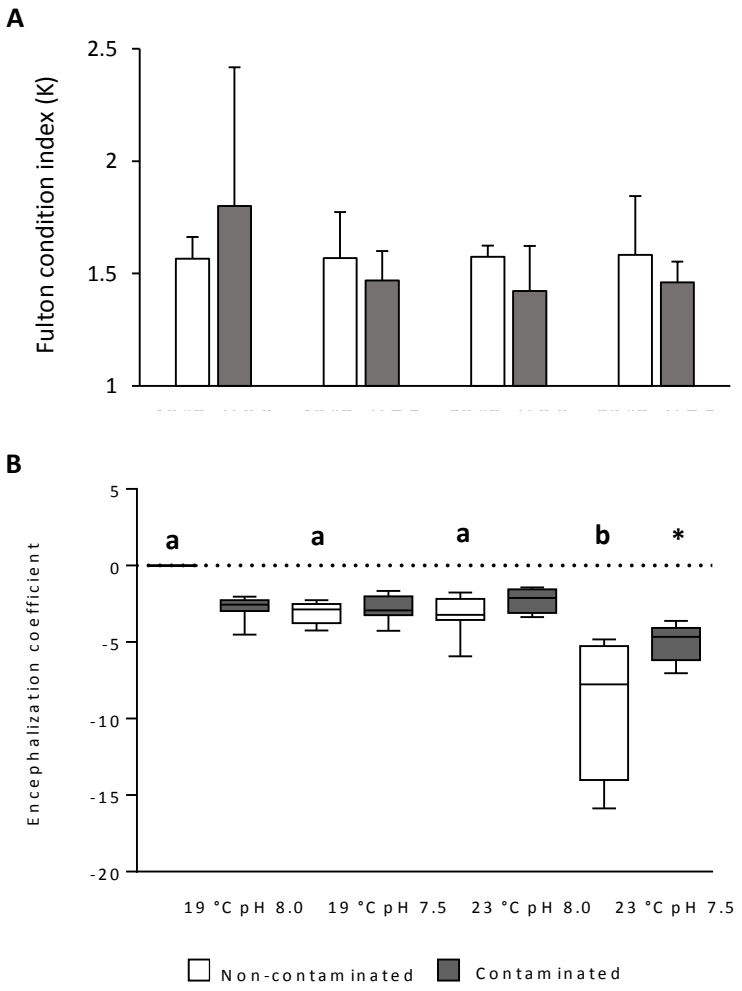
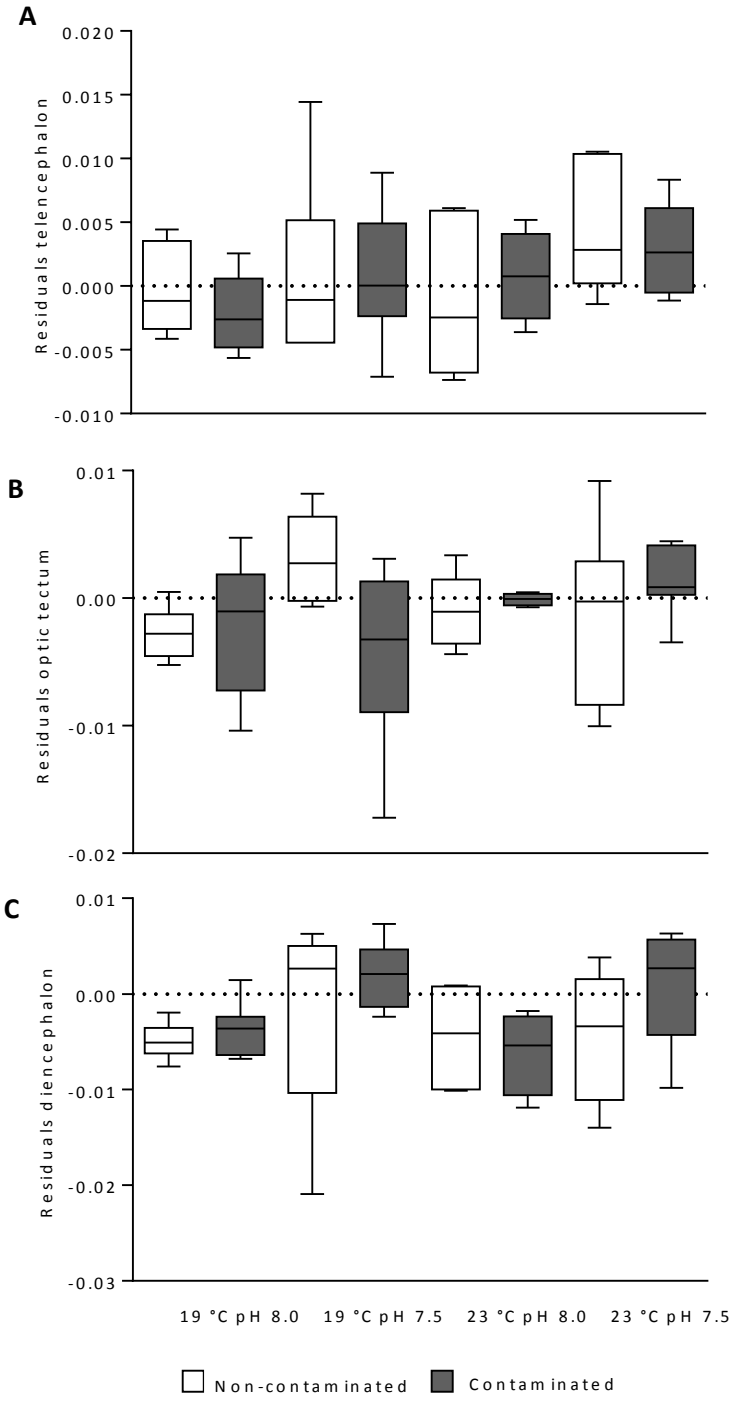


Figure 8. Impact of ocean acidification (ΔpH 0.5), warming (+4 °C) and methylmercury contamination on A) Fulton condition index and B) the encephalization coefficient of juvenile meagre (*Argyrosomus regius*). Values represent mean+s.d. Different letters represent statistical differences among treatments in the same contamination condition. Asterisk represents significant differences between contaminated and non-contaminated treatments in each temperature and pH condition.

4.3. Macro areas coefficient

Ocean warming, acidification and contamination did not cause any significant changes in the macro areas coefficient ratio between telencephalon, optic tectum, diencephalon, cerebellum (Fig. 9A, B, C and D; $P > 0.05$). Brain stem was significantly bigger than what was predicted under warming and control pH (Fig. 9E; $P < 0.05$). Between non-contaminated and contaminated warming and control pH conditions there was a significant difference in the brain stem coefficient ($P < 0.05$).



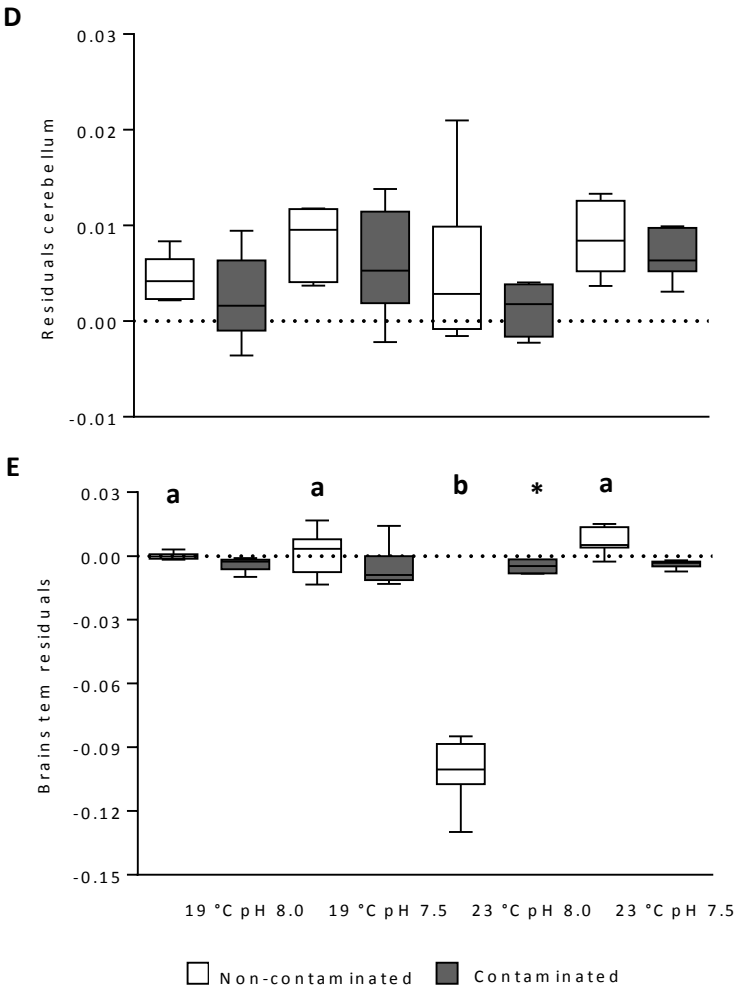


Figure 9. Impact of ocean acidification (ΔpH 0.5), warming (+4 °C) and methylmercury contamination on macroareas coefficient between the five areas of the brain A) telencephalon, B) optic tectum, C) diencephalon, D) cerebellum and E) brain stem of juvenile meagre (*Argyrosomus regius*). Values represent mean+s.d. Small letters represent statistical differences among treatments in the same contamination condition. Asterisk represents significant differences between contaminated and non-contaminated treatments in each temperature and pH condition.

4.4. Brain macro areas acetylcholinesterase activity

Ocean warming and acidification did not affect significantly AChE activity in all five meagre brain macro areas under non-contaminated conditions (Fig. 10; $P > 0.05$). However, contamination induced a significant increase in AChE activity in all areas (Fig. 10; $P < 0.05$). In the telencephalon, warming and acidification revealed an increase in AChE (1575.01 nmol min^{-1} mg^{-1} protein) compared to the activity under the same temperature condition and control pH (830.96 nmol min^{-1} mg^{-1} protein) (Fig. 10A; $P < 0.05$). Moreover, AChE in the optic tectum increased significantly under the most drastic scenario (23 °C and pH 7.5) (1434.15 nmol min^{-1} mg^{-1} protein) (Fig. 10B; $P < 0.05$). In diencephalon, AChE decreased under the warming and control pH contaminated treatment (962.35 nmol min^{-1} mg^{-1} protein) in comparison with the same temperature but hypercapnia treatment (1531.92 nmol min^{-1} mg^{-1} protein) (Fig. 10C; $P < 0.05$). In contrast, in the cerebellum, neither temperature nor pH affected the enzyme response to contamination (Fig. 10D, $P > 0.05$). Under the contaminated scenario, warming and hypercapnia increased AChE activity in brain stem (1837.70 nmol min^{-1} mg^{-1} protein) in comparison with control temperature and pH, and control temperature and hypercapnia treatments (1376.38 nmol min^{-1} mg^{-1} protein and 1375.70 nmol min^{-1} mg^{-1} protein, respectively) (Fig. 10E; $P < 0.05$).

Only the non-contaminated control temperature and pH and warming and control pH treatments showed significant differences between the five brain macro areas AChE activities under the same treatment. Under contaminated conditions, only the warming and hypercapnia condition did not reveal significant differences in the AChE levels of the different brain macro areas under the same treatment. Under the non-contaminated temperature and pH control condition, AChE was higher in the diencephalon (243.32 nmol min^{-1} mg^{-1} protein) than in the brain stem (141.08 nmol min^{-1} mg^{-1} protein) (Fig. 10; $P < 0.05$). In the non-contaminated condition, under warming and control pH, telencephalon and cerebellum had lower AChE activity (125.11 nmol min^{-1} mg^{-1} protein and 117.45 nmol min^{-1} mg^{-1} protein, respectively) than diencephalon and brain stem (206.88 nmol min^{-1} mg^{-1} protein and 217.61 nmol min^{-1} mg^{-1} protein, respectively) (Fig. 10; $P < 0.05$). Under the contaminated control temperature and pH condition, cerebellum activity was significantly lower (794.28 nmol min^{-1} mg^{-1} protein) than in the brain stem (1376.39 nmol min^{-1} mg^{-1} protein) (Fig. 10; $P < 0.05$). Under the control temperature and hypercapnia contaminated condition, diencephalon AChE activity (1475.72 nmol min^{-1} mg^{-1} protein) was statistically higher than in the cerebellum (931.20 nmol min^{-1} mg^{-1} protein) (Fig. 10; $P < 0.05$). Lastly, under the warming and control pH scenario, telencephalon, diencephalon and cerebellum had lower AChE

activity (831.00 nmol min^{-1} mg^{-1} protein, 962.35 nmol min^{-1} mg^{-1} protein and 801.95 nmol min^{-1} mg^{-1} protein, respectively) than brain stem (1461.22 nmol min^{-1} mg^{-1} protein) (Fig. 10; $P < 0.05$).

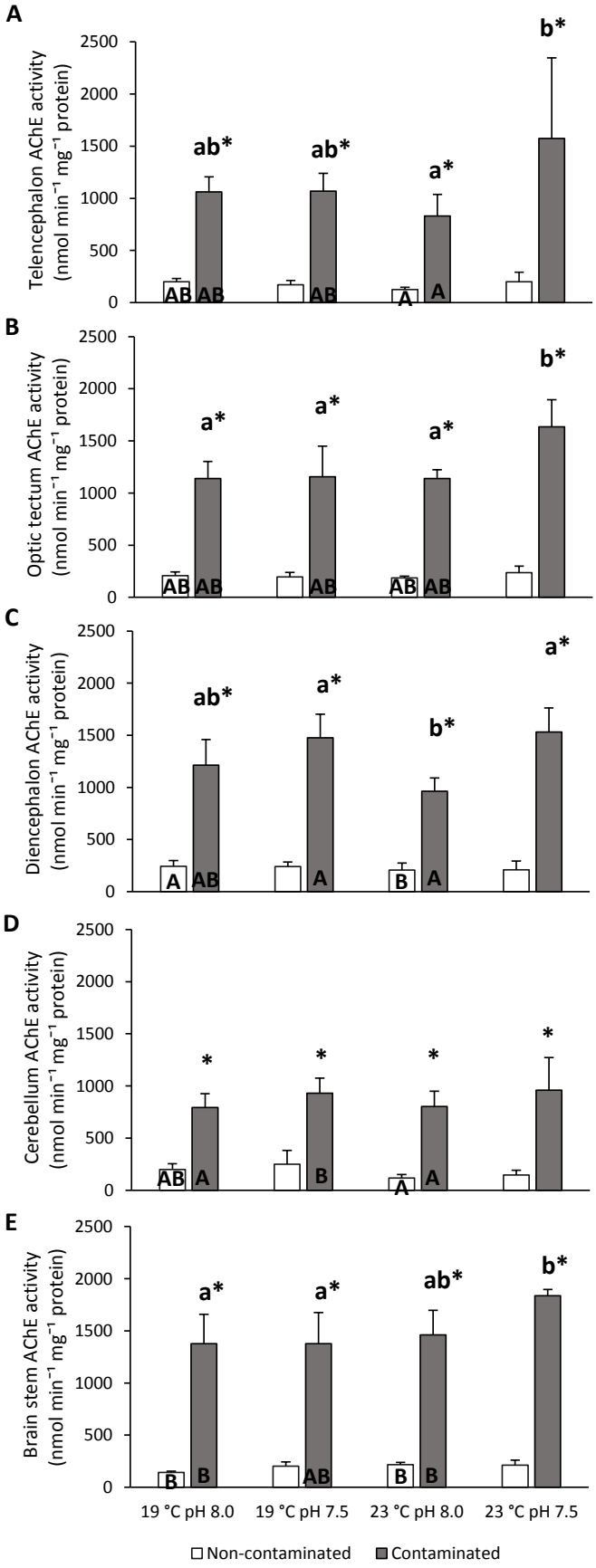


Figure 10. Impact of ocean acidification (ΔpH 0.5), warming (+4 °C) and methylmercury contamination on the acetylcholinesterase activity on the five areas of the brain A) telencephalon, B) optic tectum, C) diencephalon, D) cerebellum and E) brain stem of juvenile meagre (*Argyrosomus regius*). Values represent mean+s.d. Small letters represent statistical differences among treatments in the same contamination condition. Asterisk represents significant differences between contaminated and non-contaminated treatments in each temperature and pH condition. Capital letters represent significant differences between tissues in the same treatment.

4.5. Heat shock response and lipid peroxidation

Water temperature, pH and the usage of a non-contaminated/contaminated food source had distinct effects on HSP70/HSC70 expression in different tissues. Only muscle displayed significant differences (Fig. 11B; $P < 0.05$), i.e. an increase in the HSP content occurred under both warming and control pH conditions (5.55 ng mg^{-1} protein in the non-contaminated condition and 6.06 ng mg^{-1} protein in the contaminated condition) when compared to control conditions (19 °C pH 8.0) (3.76 ng mg^{-1} protein in the non-contaminated condition and 3.51 ng mg^{-1} protein in the contaminated condition). However, there was a significant drop in HSP content, under warming and hypercapnia conditions (23 °C pH 7.5) in non-contaminated tissue (3.94 ng mg^{-1} protein) (Fig. 11B; $P < 0.05$).

When comparing different tissues in the same condition it is evident that the HSP content changes (Fig. 11). Under control non-contaminated and contaminated conditions, HSP was statistically higher in liver (5.85 ng mg^{-1} protein in the non-contaminated condition and 6.68 ng mg^{-1} protein in the contaminated condition) than in gills (3.47 ng mg^{-1} protein in the non-contaminated condition and 3.89 ng mg^{-1} protein in the contaminated condition) and muscle (3.76 ng mg^{-1} protein in the non-contaminated condition and 3.51 ng mg^{-1} protein in the contaminated condition) ($P < 0.05$; Fig. 11). At warming and control pH scenario, HSP's were significantly higher in the muscle (5.55 ng mg^{-1} protein) when compared to gills and liver (3.19 ng mg^{-1} protein and 3.60 ng mg^{-1} protein, respectively) ($P < 0.05$; Fig. 11). Under the non-contaminated warming and hypercapnia scenario, HSP's were statistically higher in the muscle and liver (3.94 ng mg^{-1} protein and 4.77 ng mg^{-1} protein, respectively) ($P < 0.05$; Fig. 11).

Regarding MDA levels, no significant differences were observed between treatments ($P > 0.05$; Fig 11D).

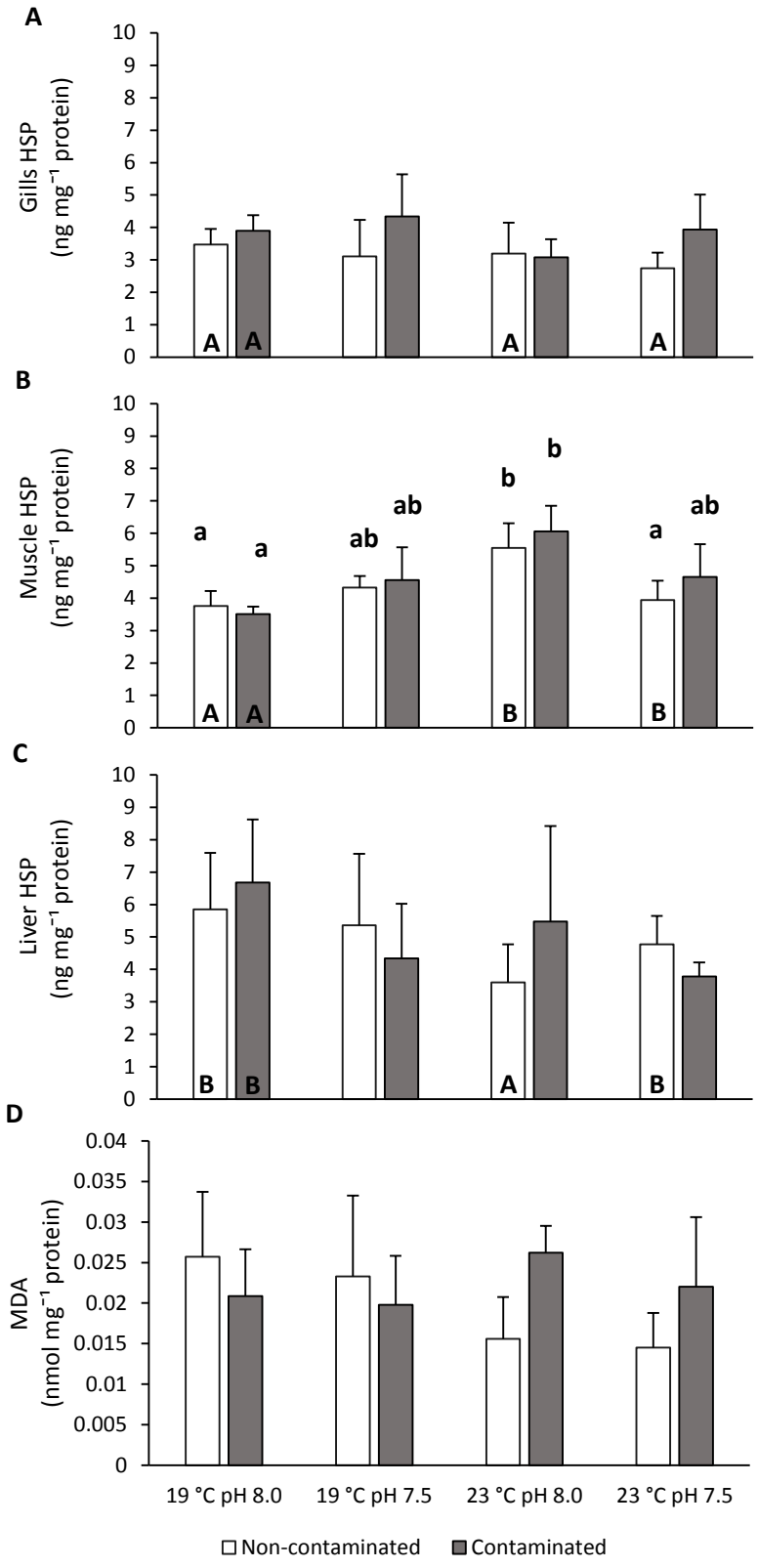


Figure 11. Impact of ocean acidification (ΔpH 0.5), warming (+4 °C) and methylmercury contamination on HSP in A) gills, B) muscle and C) liver and on D) muscle MDA concentration in juvenile meagre (*Argyrosomus regius*). Values represent mean+s.d. Small letters represent statistical differences among treatments in the same contamination condition. Capital letters represent significant differences in HSP between tissues in the same treatment.

4.6. Antioxidant enzymes activities

The impact of ocean warming, acidification and contamination on antioxidant enzymes (GST, CAT and SOD) in the muscle of juvenile *Argyrosomus regius* is shown in Figure 12.

GST activity did not change with contamination (Fig. 12A; $P > 0.05$). Warming caused an increase in GST activity ($P < 0.05$). Furthermore, under contamination, GST was statistically the highest under warming and control pH scenario when compared with warming and acidification, with values ranging from 9.47 nmol min^{-1} mg^{-1} protein to 6.05 nmol min^{-1} mg^{-1} protein, respectively, ($P < 0.05$).

Catalase activity did not display significant differences in non-contaminated treatments (Fig.12B; $P > 0.05$), however when contaminated with MeHg, meagre CAT activity statistically increased from 4.22 to 6.64 nmol min^{-1} mg^{-1} protein under the control temperature and hypercapnia scenario ($P < 0.05$).

SOD activity did not show any significant differences under non-contaminated treatments (Fig.12C; $P > 0.05$). In contrast, in contaminated conditions, SOD was significantly higher under the control temperature and acidification treatment ($P < 0.05$).

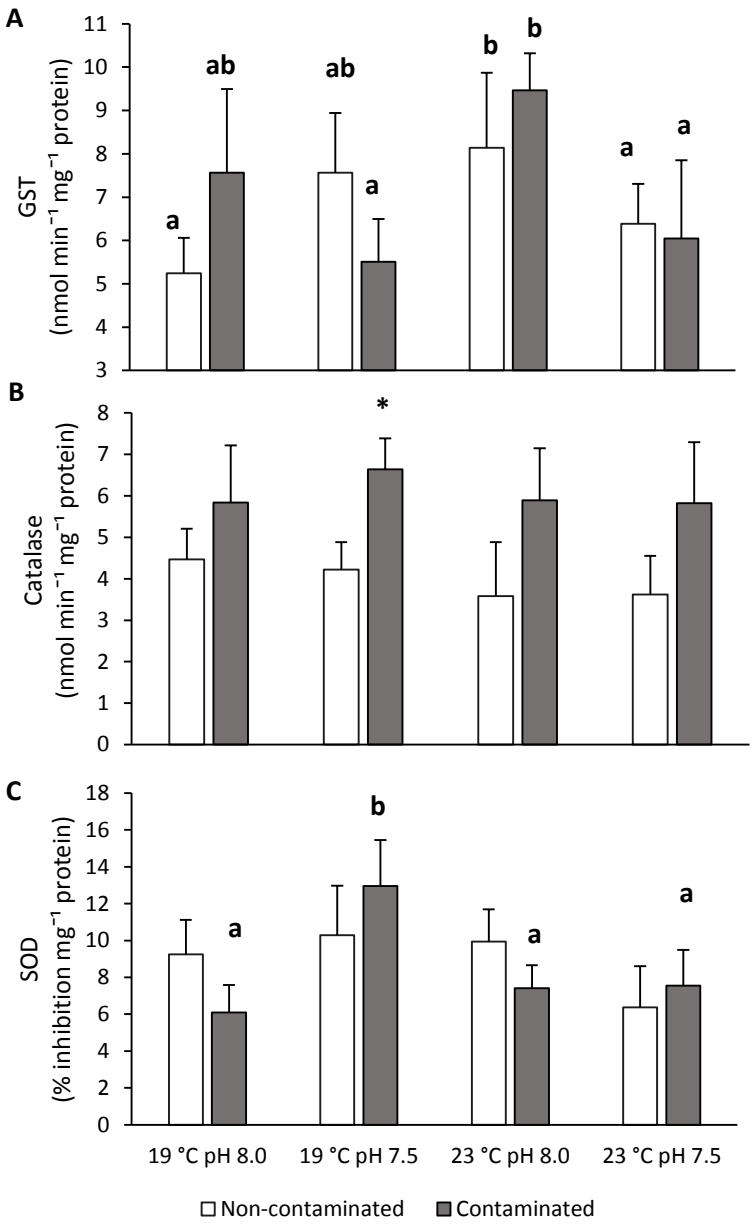


Figure 12. Impact of ocean acidification (ΔpH 0.5), warming (+4 °C) and methylmercury contamination in the A) GST activity, B) Catalase activity and C) SOD activity in the muscle tissue of juvenile meagre (*Argyrosomus regius*). Values represent mean+s.d. Different small letters represent statistical differences among treatments in the same contamination condition. Asterisk represents significant differences between contaminated and non-contaminated treatments in each temperature and pH condition.

5. Discussion

5.1. Total mercury accumulation

Aquatic organisms can accumulate mercury from water, through gills, or from feed ingestion (Jernelöv and Lann, 1971). Here, the contamination occurred through feed intake. Furthermore, mercury accumulation in fish depends on the water quality of their habitat (temperature, pH, alkalinity, etc...) (Harris and Bodaly, 1998; Ponce and Bloom, 1991; Wren et al., 1991). Temperature can influence mercury bioaccumulation in fish (Harris and Bodaly, 1998) with evidence from killifish to rainbow trout (Dijkstra et al., 2013; MacLeod and Pessah, 1973). Moreover, several studies have demonstrated that shifts in pH can also influence mercury accumulation in fish. For example, Scheuhammer and Graham, (1999) revealed that fish from an acidic lake accumulated more mercury compared to fish from a neutral lake, thus concluding that pH is negatively correlated with mercury concentrations in freshwater fish (Ponce and Bloom, 1991; Wren et al., 1991) and also invertebrates (Wren and Stephenson, 1991). In the present study, warming increased mercury accumulation in fish compared with the control condition, though only significantly in gills and muscle. Despite the evidence that low pH might increase mercury accumulation in freshwater fish (Haines et al., 1992; Ponce and Bloom, 1991), the current findings did not reflect this pattern. It is worth noting that possible evolutionary adaptations might influence the different results observed compared to the previous studies with freshwater.

Affinity to metal accumulation can differ between fish organs. Muscle is usually the organ with less affinity to metals (Jezierska and Witeska, 2006) compared to liver where metals accumulate at higher levels (Jezierska and Witeska, 2006), as the latest tissue plays a major role in accumulation and detoxification of metals (Gbem et al., 2001; Wagner and Boman, 2003). Mercury levels are usually higher in the liver also because this contaminant is absorbed at the intestine from the feed, being an important route for mercury uptake, and then transported to the liver through the hepatic portal system (Foster et al., 2000). Indeed, in this study muscle accumulated less mercury in most treatments, whereas liver had the highest mercury accumulation in most treatments. Higher concentrations of Hg have been found in liver in striped bass, catfish, and tilapia in comparison with Hg concentrations in skeletal muscle, liver, blood, gonad, brain, gill, and heart of these species (Cizdziel et al., 2003). Brain is an organ that usually also shows high metal accumulation (Jezierska and Witeska, 2006). Gills displayed lower mercury accumulation. This tissue is very sensitive to metal accumulation when waterborne metal exposure occurs (De Domenico et al., 2011). However, in this study

contamination was made through feed intake, which may have caused gills to accumulate less mercury under the warming and acidification condition. It is known that the interaction between warming and acidification can have additive or antagonistic effects on gills mercury uptake (Byrne and Przeslawski, 2013), likely justifying the results obtained in this study.

5.2. Fulton condition and survival

In the present study, meagre did not demonstrated any changes in survival and Fulton condition under ocean warming, acidification and contamination. Nonetheless, MacLeod and Pessah, (1973) revealed that mortality rate, due to mercury exposure, is linearly related to temperature in freshwater fish. In zebrafish, survival is affected by mercury too (Penglase et al., 2014). Moreover, mercury concentrations are negatively correlated with Fulton condition in wild river fish populations (Pyle et al., 2005). Such results indicate that fish species can respond differently to environmental changes and contamination levels. In fact, meagre is a very resilient species and can easily adapt to environmental alterations (Monfort, 2010), which may explain the absence of such expected effects.

5.3. Encephalization

Teleost fish brain grows continuously throughout fish lifetime (Ekström, 1994; Kotrschal et al., 1998). Hence, due to brain plasticity this organ can be affected by diverse environmental changes that occur during fish life (Kihslinger and Nevitt, 2006). It has been reported that brain size can be influenced by habitat complexity (Pollen et al., 2007; Shumway, 2010) and type of diet (Gonzalez-Voyer et al., 2009) in African cichlid fish. Rearing conditions can also affect brain size in Atlantic salmon, as fish reared in stream conditions can develop smaller brains compared with fish in hatcheries (Näslund et al., 2012). Kotrschal et al., (2012) also noted that animals reared in hatchery developed larger brains. In contrast, Marchetti and Nevitt, (2003) observed that brains of hatchery reared rainbow trout are smaller than wild fish. Furthermore, hypoxia can also have effects in brain size. Chapman and Hulen, (2001) showed that brain size of two mormyrids species from extremely hypoxic waters are significant smaller than the brain of two open-water species from well oxygenated habitats. Furthermore, Chapman et al., (2008) demonstrated that brain size is smaller in African cichlids raised under hypoxia.

Yu et al., (2014) formulated a framework that identifies temperature as the most critical factor to brain enlargement and shows that fish in warmer habitats have larger brain than those living in colder waters. Gillooly and McCoy, (2014) also discussed that brain size increases with temperature in vertebrates, suggesting that higher temperature

can increase energy supply by speeding biochemical reaction rates and allowing fish to keep more brain tissue. In the present study, this effect did not occur under the warming condition. However, brain was larger under the warming and acidification treatment on both non-contaminated and contaminated treatments. The interaction between warming and acidification can have additive or antagonistic effects in marine organisms (Byrne and Przeslawski, 2013; Crain et al., 2008). Thus, hypercapnia may have enhanced the effect of temperature on brain and increased its size. Meagre may have invested more energy in brain development instead of relocating such energy to other functions as a response to the synergistic condition, which could be advantageous since fish benefits with larger brains as this tissue has innumerous important functions (Crispo and Chapman, 2010). Also, larger brains may promote the development of different behavioural patterns helping fish dealing more successfully with new environmental challenges (Sol, 2009). So the brain increase observed under the warming and acidification treatment may have been an attempt of meagre to adapt to the environmental conditions.

Mercury can also cause brain lesions in fish (Berntssen et al., 2003). Encephalization coefficient was significantly different between the non-contaminated scenario (with a significantly larger brain mass) and the contaminated one, suggesting that mercury significantly affected brain investment.

5.4. Brain macro areas

Environment conditions can affect brain structures, including its size and volume (Kihslinger et al., 2006; Kotrschal et al., 2012). Kihslinger et al., (2006) reported that olfactory bulb and telencephalon are larger in wild fish compared to hatchery-reared fish in a single generation of Chinook salmon. Different rearing conditions also cause an increase in the cerebellar area of salmon alevin (Kihslinger and Nevitt, 2006). Furthermore, different hatchery-rearing practices influence different brain structures like the optic tectum and the telencephalon in rainbow trout (Marchetti and Nevitt, 2003). Thus, it is possible to see different responses in brain structures to environmental stressors. In the present study, brain stem was the only brain area affected by environmental conditions. This structure controls sensory systems (except smell and sight) and governs certain somatic and visceral functions. It also controls respiration and osmoregulation in bony fish (Helfman et al., 2009).

Fish can accumulate mercury in specific areas of the brain. Rouleau et al., (1999) showed that rainbow and brown trout exposed to waterborne mercury can accumulate this metal in several brain areas, for example, in cerebellum and optic tectum. This suggests that in these fish species waterborne Hg was taken by water-exposed receptor

cells of sensory nerves such as: receptors of lateral line system, cutaneous sensory cells, and receptor cells of taste buds in oral mucosa and then transferred to the different areas of the brain through axonal transport (a physiological process for the transport of dissolved neuronal constituents along nerve axons). In this study, contamination was made through feed intake, which can lead to an uptake of mercury through taste buds and subsequently transported to the brain. However, contamination did not affect brain areas in the present study, although brain have shown high levels of mercury contamination, probably due to the fact that meagre is a resilient species and easily adaptable to different environmental conditions (Monfort, 2010).

As previously said, high temperature is linked to larger brains (Gillooly and McCoy, 2014; Yu et al., 2014). In this study, warming affected brain stem size, and caused an enlargement in this structure, likely as a response to the natural increase in metabolic rates, energy supply, and biochemical reaction rates that occur at higher temperatures (Gillooly and McCoy, 2014), favouring an increased investment in brain stem.

5.5. Acetylcholinesterase (AChE) activity

Diverse AChE responses have been reported when exposed to different toxic compounds (Richetti et al., 2011; Romani et al., 2003). Although temperature can alter AChE activity (Baldwin, 1971; Hogan, 1971), future temperature conditions did not affect AChE response in meagre. It has been described that ocean acidification can alter GABA function in fish brain (Nilsson et al., 2012). GABA is an important neurotransmitter in the central nervous system (CNS) as well as acetylcholine (Ach), which is hydrolyzed by AChE. Hence, it is expected that acidification alter AChE activity in meagre. However, this was not observed. Meagre is a high resilient species, which may explain why warming and acidification under non-contaminated treatments did not affect significantly AChE activity.

Here, it is also shown that AChE activity in the five brain areas was remarkably higher in contaminated meagre. In contrast, several studies have shown a decrease in fish AChE in the presence of mercury and other metals (Gill et al., 1990; Richetti et al., 2011). AChE also seems to have different responses to other contaminants. In fact, an increase in AChE activity has been demonstrated in fish exposed to copper, zinc and ethanol (Dethloff et al., 1999; Rico et al., 2007; Romani et al., 2003).

Interestingly, in vertebrates it has already been reported that the exposure to MeHg compromise the cerebral dopaminergic and serotonergic systems according to the brain area (Castoldi et al., 2006). This suggests that the neurotransmitter Ach may also be affected by MeHg, likely explaining the variation in AChE activities among the

different brain macro areas for the same treatment, as each area can have a distinct way to respond to environmental stresses. Except for cerebellum and brain stem, under contaminated treatments, the warming and acidification condition showed higher AChE activity than the warming treatment. The synergism between the two factors probably exacerbated the effect of contamination, enhancing AChE activity in meagre brain. Moreover, this synergism has also been demonstrated to increase AChE levels in sharks (Rosa et al., 2015 under submission). In optic tectum and brain stem a difference between the effect of acidification and the synergistic condition (high temperature and low pH) was observed. As expected, under this condition, AChE levels were higher, showing once again that synergism aggravated the effect of contamination.

It is worth noting that AChE can be involved in the regulation of apoptosis (Jin et al., 2004; Zhang et al., 2002). Since mercury can induce apoptosis in fish (Drevnick et al., 2006; Sarmento et al., 2004), the increase in AChE activity in contaminated treatments likely indicate the initiation of this mechanism (De Domenico et al., 2013; Jin et al., 2004; Zhang et al., 2002). Furthermore, oxidative stress increases AChE activity (Melo et al., 2003) and Hg is responsible for oxidative stress in fish brain (Berntssen et al., 2003). Therefore, Hg may have triggered oxidative stress in meagre brain, leading to an increase in AChE activity.

5.6. Oxidative stress

High temperatures might lead to the formation of reactive oxygen species (ROS) (Abele et al., 2002; Lesser, 2006). HSP are one of the molecules that protect and control ROS formation (Jacquier-Sarlin et al., 1994). These proteins help to repair, refold and eliminate damaged or denatured proteins (Sokolova et al., 2011). In non-contaminated and contaminated warming conditions, HSP in muscle showed an increase, demonstrating a stress response in fish. Acidification did not change HSP response indicating that the effect of temperature on HSP is stronger than acidification. Under the non-contaminated condition, the synergism between high temperature and low pH diminished the heat effect on HSP, demonstrating that acidification exhibit an antagonistic effect.

In muscle, contamination did not affect HSP response. Gills are usually the most affected tissues when fish is exposed to contamination, particularly when contamination occurs through water (Farag et al., 1994). In the present study, the contamination occurred via diet, and therefore gills were less affected (revealing the smallest HSP response) when compared to liver and muscle. Liver did not exhibit any differences in HSP response among the treatments, despite displaying the highest levels of HSP in

comparison with other tissues, likely since liver plays a major role in the detoxification of xenobiotics (Williams et al., 1996).

Lipid peroxidation is one of the most common cellular injury mechanisms (Lesser, 2006). MDA concentration has not changed in any treatment, indicating that ROS did not have an effect on meagre lipids (peroxidation). Thus, it is possible to conclude that HSP response and antioxidant enzymes activities seemed to work efficiently against cellular injuries.

Antioxidant enzymes are another defense mechanism against oxidative stress. Among them, GST is involved in the protection against xenobiotics (Wang et al., 2000), SOD converts superoxide (O_2^-) into hydrogen peroxide (H_2O_2) and CAT converts H_2O_2 into water (H_2O) and oxygen (O_2) (Lesser, 2006). Antioxidant enzymes generally increase in response to ROS production, which in turn is increased with warming (Abele et al., 2002; Lesser, 2006). It has been reported that GST increases in response to warming in marine animals, such as sole post-metamorphic larvae (Pimentel et al., 2015) and tropical and temperate shrimp (Rosa et al., 2014a). Hence, as expected, meagre GST increased at 23 ^{0}C, in order to regulate ROS production. Similarly to HSP, significant differences were only observed in the warming treatment, showing once more that warming has a more drastic effect on meagre than acidification. The synergism between warming and acidification decrease GST activity in the contaminated treatment, indicating that once again acidification has an antagonistic effect in temperature, decreasing its negative effect in GST activity.

Catalase generally increased in response to contamination. However this effect was only significant under the acidification and control temperature treatment. Previous studies reported contradictory responses of catalase to metal contamination. For instance, silver decrease CAT activity, whereas cadmium, zinc and chromium increased CAT activity in the liver of freshwater fish (Atli et al., 2006). In another study with the same fish model, liver CAT activity increased with cadmium and lead exposures, but was inhibited by zinc (Atli and Canli, 2007). Furthermore, Mieiro et al., (2011) observed CAT depletion in mercury contaminated meagre. In the present study, CAT activity increased in samples from contaminated treatments, likely to control ROS production. Also, the absence of significant differences between the various non-contaminated treatments in CAT activity may also suggest that meagre is a tolerant species to high temperatures and acidification.

In the present study, SOD did not exhibit significant differences between non-contaminated and contaminated treatments. This enzyme activity only revealed differences between contaminated treatments, e.g. acidification increased SOD activity. It is not well studied if hypercapnia causes ROS increase in fish. However, Tomanek et

al., (2011) reported that hypercapnia may enhance oxidative stress in oysters. Although acidification did not have an effect on GST, SOD activity increased under the low pH contaminated treatment possibly to protect fish from ROS. This enzyme can also increase in response to MeHg, suggesting an adaptive response of the redox defense system (Berntssen et al., 2003). So, contamination and acidification may have triggered a defense response in meagre, causing the increase in SOD activity.

6. Final remarks

The present study shows that meagre will not suffer major consequences under the expected future ocean contaminated conditions. Nonetheless, here it is shown that warming will be the most relevant factor that will increase mercury bioaccumulation in meagre. Some variables were only affected in contaminated treatments, especially AChE. Although meagre suffered some physiological alterations under these conditions, meagre responses were not apparently harmful for fish. These results suggest that meagre will be able to adapt to the predicted future ocean conditions even under mercury contamination. However, future studies will be necessary to comprehensively understand how marine biota will respond to the combined effects of future warming, acidification and pollution.

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Supplemental material

Table S1. Seawater carbonate chemistry data for the different climate change and contamination scenarios.

	Non-contaminated			
	19 °C		23 °C	
	8.0	7.5	8.0	7.5
measured				
Temperature (°C)	19.4 ± 0.4	19.0 ± 0.4	23.5 ± 0.1	23.3 ± 0.1
Salinity	35.0 ± 0.6	35.0 ± 0.3	35.0 ± 0.5	35.0 ± 0.4
pH_T	7.99 ± 0.02	7.51 ± 0.19	7.97 ± 0.04	7.49 ± 0.11
A_T (µmol kg^{-1} SW)	2002.10 ± 94.81	2176.81 ± 71.07	1971.85 ± 66.04	2093.13 ± 53.90
calculated				
pCO_2 (ppm)	408.46 ± 20.10	1512.40 ± 50.02	417.2 ± 14.56	1527.82 ± 39.93
C_T (µmol kg^{-1} SW)	1872.45 ± 170.67	2139.46 ± 70.79	1829.16 ± 175.05	2098.78 ± 119.37
Ω Ar	2.27 ± 0.21	0.88 ± 0.03	2.52 ± 0.24	1.00 ± 0.06

	Contaminated			
	19 °C		23 °C	
	8.0	7.5	8.0	7.5
measured				
Temperature (°C)	19.1 ± 0.4	19.0 ± 0.4	23.0 ± 0.2	23.3 ± 0.2
Salinity	34.8 ± 0.4	35.0 ± 0.3	35.0 ± 0.5	35.1 ± 0.5
pH_T	7.97 ± 0.06	7.53 ± 0.13	8.00 ± 0.04	7.52 ± 0.29
A_T (µmol kg^{-1} SW)	1995.02 ± 83.55	2329.40 ± 92.52	2046.84 ± 54.98	2103.69 ± 90.60
calculated				
pCO_2 (ppm)	421.92 ± 18.34	1532.54 ± 61.65	402.67 ± 11.29	1450.64 ± 63.45
C_T (µmol kg^{-1} SW)	1850.86 ± 123.61	2283.66 ± 91.88	1849.83 ± 98.86	2045.80 ± 89.51
Ω Ar	2.15 ± 0.14	0.99 ± 0.04	2.65 ± 0.14	1.03 ± 0.04